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Increased atherosclerosis in mice with increased vascular biglycan content

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Abstract

Objective—The response to retention hypothesis of atherogenesis proposes that atherosclerosis is initiated via the retention of atherogenic lipoproteins by vascular proteoglycans. Co-localization studies suggest that of all the vascular proteoglycans, biglycan is the one most closely co-localized with LDL. The goal of this study was to determine if over-expression of biglycan in hyperlipidemic mice would increase atherosclerosis development.

Methods—Transgenic mice were developed by expressing biglycan under control of the smooth muscle actin promoter, and were crossed to the LDL receptor deficient (C57BL/6 background) atherosclerotic mouse model. Biglycan transgenic and non-transgenic control mice were fed an atherogenic Western diet for 4-12 weeks.

Results—LDL receptor deficient mice overexpressing biglycan under control of the smooth muscle alpha actin promoter had increased atherosclerosis development that correlated with vascular biglycan content.

Conclusion—Increased vascular biglycan content predisposes to increased lipid retention and increased atherosclerosis development.

Keywords

atherosclerosis; mice; biglycan; LDL retention

Introduction

As outlined in the “Response to Retention” hypothesis, atherosclerosis is initiated by the retention of atherogenic lipoproteins in the subendothelial space by their ionic interactions

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with vascular wall proteoglycans¹. Several studies have demonstrated that biglycan is the proteoglycan most co-localized with apolipoprotein (apo)B in both mouse and human atherosclerosis²⁻⁵. Mice expressing proteoglycan-binding-defective apoB had decreased atherosclerosis, demonstrating the role of proteoglycan-lipoprotein interactions in atherosclerosis⁶. In previous studies we demonstrated that angiotensin (ang)II up-regulated vascular biglycan content which caused increased LDL binding and increased atherosclerosis following a high fat/high cholesterol diet². However, angII has a number of pro-atherogenic properties (for review see⁷) thus this is not definitive proof that vascular biglycan content directly affects atherosclerosis development. The goal of this study was to determine if increased vascular biglycan content in a hyperlipidemic mouse led to increased atherosclerosis.

Materials and Methods

All animal care and experimental procedures were approved by and performed in accordance with the University of Kentucky Animal Care Committee and conformed to Public Health Service policy on the humane care and use of laboratory animals. Biglycan transgenic mice were derived directly in the C57Bl/6 genetic background from a construct generously provided by Sasamura et al in which the human biglycan gene was ligated downstream from the smooth muscle alpha actin promoter⁸. The construct was sent to Xenogen Biosciences who performed the microinjections. Three male founders were provided initially but appeared to be infertile as no pups resulted despite a variety of breeding strategies. Thus, one male founder was shipped to Charles River Laboratories where in vitro fertilization was performed using C57BL/6 females. The resulting offspring were transferred back to University of Kentucky animal facility and were crossed with LDL receptor deficient mice (C57Bl/6 background). The resulting offspring from this cross were identified by PCR analysis using the following primers: forward primer: 5'-TCC TGG GAC TTC ACC CTG GA-3' and reverse primer: 5'-TTG AAG TCA TCC TTG CGG AG-3'. Mice positively identified with the biglycan transgene were bred together and labeled transgenic. Mice that did not have the biglycan transgene were bred together and were labeled as the wild-type control.

All mice were housed in a temperature-controlled facility with 12 hour light/dark cycles and fed normal rodent chow ad libitum with free access to water. At 10-12 weeks of age the diet was changed to Western diet (0.15% cholesterol and 21% fat; Harlan laboratories catalog number 88137, Madison, WI) for 4 or 12 weeks. At the end of the study blood and major organs including major blood vessels were collected for metabolic assays and measurement of atherosclerosis. Total cholesterol and triglyceride levels were determined using enzymatic cholesterol assay kits (Wako Chemical Co, Richmond, VA), and lipoprotein distribution was measured by fast performance liquid chromatography (FPLC). Plasma TGFβ was measured using the TGFβ1 Emax® ImmunoAssay system (Promega, Madison, WI) on acid activated samples. Atherosclerosis was quantified in the aortic sinus, aortic intimal surface and brachiocephalic artery as previously described^{2, 9}. Aortic root sections were double labeled for biglycan (R and D Systems, Minneapolis, MN) and apoB (BioDesign International, Saco, ME) and visualized using confocal microscopy as previously described⁹. Brachiocephalic artery sections were stained for elastin using a modified Verhoeff Van

Gieson Elastic stain kit, (Sigma, St. Louis, MO) to measure medial area. Medial area was defined as the difference between internal elastic lamina area and external elastic lamina area. Total protein was extracted from carotids by TRIZOL reagent and immunoblotted for biglycan (R and D Systems, Minneapolis, MN) or actin (Sigma, St. Louis, MO; AF2066) as previously described⁹. Immunoblot quantification was performed using ImageJ software (National Institutes of Health) and is expressed as relative biglycan density divided by actin density. Statistical differences were assessed by two-way ANOVA to assess effects of genotype and gender or by two-tailed t-test. Regression analysis was performed to compare biglycan content with atherosclerotic lesion area. $P < 0.05$ was considered statistically significant.

Primary vascular smooth muscle cells were isolated from ~3 week old mice and grown to confluence without passaging, then incubated with Alexa-fluor labeled LDL² for 2 hours at 4°C. Cells were washed twice in cold PBS then fixed in 4% formaldehyde, imaged and quantified using ImageJ software. LDL binding is expressed as Alexa-fluor intensity normalized to cell number.

Results

LDL receptor null mice expressing the human biglycan transgene and transgene negative controls were fed an atherogenic Western diet for 12 weeks. As expected, male mice gained more weight than female mice, but there was no effect of genotype on body weight (wildtype males: 41.1 ± 1.4 , wildtype females: 27.9 ± 1.1 , transgenic males: 39.3 ± 1.7 , transgenic females: 28.1 ± 0.9 g at study end). Total cholesterol (wildtype males: 1658 ± 62 , wildtype females: 1379 ± 72 , transgenic males: 1556 ± 61 , transgenic females: 1299 ± 38 mg/dl) was significantly different between genders ($P=0.003$) but there was no effect of the transgene; triglycerides (wildtype males: 1385 ± 170 , wildtype females: 461 ± 58 , transgenic males: 917 ± 96 , transgenic females: 485 ± 31 mg/dl) were similarly different between genders ($P<0.001$) and there was a significant difference between genotypes ($P=0.005$). However, lipoprotein profiles determined by FPLC did not differ between genotypes (Fig. 1A). TGF- β concentrations did not differ between either genotype or gender (average TGF- β concentration was 6410 pg/ml). There was no difference between genders for atherosclerosis so genders were combined for analyses of atherosclerosis. Atherosclerotic lesion area measured at the aortic sinus and en face aorta was significantly increased in biglycan transgenic mice compared to controls (Fig. 1B showing aortic sinus, 1.3 fold increase, $P=0.006$; Fig. 1C showing aortic intimal surface, 1.25 fold increase, $P=0.05$). Brachiocephalic artery atherosclerotic lesion area did not differ between groups (Fig. 1D). To determine if lipid retention was more rapid in biglycan transgenic mice, a subset of mice were killed after 4 weeks of diet. There was minimal atherosclerosis at this early time-point but brachiocephalic artery atherosclerosis was increased by 1.3 fold (not shown) in transgenic (average lesion area $25,769 \pm 6,532 \mu\text{m}^2$) compared to wildtype mice (average lesion area $19,473 \pm 6,327 \mu\text{m}^2$), although this did not reach statistical significance, likely due to the small n. There was no difference in medial area between groups (not shown).

Biglycan was found within the media and was increased in transgenic compared to control mice (Fig. 2A). Vascular biglycan content was measured by immunoblots on protein

extracted from carotid arteries (Fig. 2B) as well as by quantification of immunostained area in the aortic sinus and was increased in transgenic compared to wildtype mice (Fig. 2C, $P<0.001$). Regression analysis showed that aortic sinus atherosclerosis correlated with carotid vascular biglycan content (Fig. 2D; $r=0.539$, $P<0.0001$) and with aortic sinus biglycan content (Fig. 2E, $r=0.330$, $P=0.03$). Aortic sinus sections were double stained for biglycan and apoB and demonstrate co-localization of biglycan and apoB in atherosclerotic lesions of biglycan transgenic mice (Fig. 2F). There was a trend towards increased LDL binding to primary vascular smooth muscle cells from transgenic mice compared to those from control mice (Fig. 2G).

Discussion

Hyperlipidemic mice overexpressing biglycan under control of the smooth muscle alpha actin promoter had increased atherosclerosis development that correlated with vascular biglycan content. Although no significant differences were found in atherosclerotic lesion area of the brachiocephalic artery at the 12 week time point, this may be due to differences in timing of lesion development and progression at different sites in the vasculature, for example due to different flow conditions, different embryological origins for the tissue, or other factors.^{10, 11} In support of this concept, we found a 1.3 fold increase in brachiocephalic artery atherosclerosis in transgenic compared to wildtype mice at the 4 week time point (albeit the 4 week study was under-powered and thus did not reach statistical significance). As expected, there were differences in lipid levels and weight between genders. Although triglyceride levels differed between genotypes, there was no difference in total cholesterol or lipoprotein profiles between genotypes. However, increased levels of triglyceride-rich lipoproteins are thought to be atherogenic, as like cholesterol-rich particles they can be trapped in the subendothelial matrix. Furthermore, triglyceride-rich lipoproteins contain considerably more cholesterol per particle than LDL particles, thus can accelerate atherosclerosis by enhanced cholesterol delivery to the vascular wall¹². However, the mechanism of triglyceride-accelerated atherosclerosis is thought to be mediated by increased vascular retention, thus the increased vascular biglycan content in this model is a major cause of the increased atherosclerosis observed.

Although all proteoglycans can bind lipoproteins *in vitro*, co-localization studies have consistently shown that biglycan is the proteoglycan most co-localized with apoB in both mouse and human atherosclerosis²⁻⁵, although in mice apoB also co-localizes with perlecan^{2, 3}. In human tissues there is a relative increase in chondroitin sulfate and dermatan sulfate proteoglycans (including biglycan) and a relative decrease in heparan sulfate proteoglycans (including perlecan) in atherosclerotic lesions compared to adjacent, non-atherosclerotic regions of the same artery^{13, 14}. Nakashima et al examined human atherosclerotic lesions of various stages and found that biglycan was present in regions of diffuse intimal thickening in the same location that early lipid retention was seen, suggesting that biglycan accumulation may pre-dispose to vascular lipid retention and atherosclerosis initiation⁴. In a previous study we tested this hypothesis utilizing angII which had been shown to up-regulate vascular biglycan¹⁵. LDL receptor deficient mice infused with angII had increased vascular biglycan content; using a perfusion assay we demonstrated that vessels with increased biglycan had increased lipid retention; upon subsequent challenge

with an atherogenic diet there was increased atherosclerosis development in the mice that had received angII compared to the control groups². The present study provides further support for the response to retention hypothesis of atherosclerosis, by demonstrating that genetically induced increases in vascular biglycan content predispose to increased atherosclerosis. Given the robust literature on lipoprotein-proteoglycan interactions we propose that over-expression of biglycan in vessels leads to increased retention of the atherogenic lipoproteins that migrate in and out of the artery wall^{16, 17} triggering early lesion development characterized by fatty streaks. Biglycan has a number of other properties that may affect its role in atherogenesis, including a role in inflammation¹⁸, regulation of growth factor availability¹⁹ and protection against hypoxia/ reoxygenation injury²⁰; thus, further study is needed to investigate these potential effects.

In this study we used a genetic approach to over-express biglycan; it is not apparent if there is genetic variation in human vascular biglycan content that could account for differences in atherosclerosis and cardiovascular disease clinically. However, we and others have investigated the regulation of biglycan expression in the vasculature and a number of potential candidates that could lead to increased vascular biglycan content have been identified. For example, TGF β robustly increases biglycan expression and increases glycosaminoglycan chain length, which increases LDL binding, at least *in vitro* (for review see²¹). TGF β is elevated in a variety of clinical settings, including diabetes^{22, 23}. In other studies, we demonstrated that the acute phase reactant serum amyloid A (SAA), which is persistently and chronically elevated in conditions of chronic inflammation such as diabetes, obesity, rheumatoid arthritis, etc (which are all associated with increased cardiovascular disease) increases vascular biglycan expression and LDL binding affinity²⁴. Other factors that have been shown to up-regulate vascular biglycan include mechanical strain²⁵, oxidized LDL²⁶, and fatty acids^{27, 28}. Thus it is certainly feasible that elevated levels of such factors could lead to increases in vascular biglycan content pre-disposing an individual to increased atherosclerosis, and thus vascular biglycan content may be a therapeutic target to reduce atherosclerosis initiation.

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Figure 1.

Overexpression of biglycan increases atherosclerosis. Biglycan transgenic (TG) and non-transgenic wildtype control (WT) LDL receptor deficient mice were fed an atherogenic Western diet for 12 weeks. Lipoprotein distribution was measured by FPLC after 12 weeks of diet (A). Atherosclerosis was measured in the aortic sinus (B), en face aortic intimal surface (C), and brachiocephalic artery (D). Each symbol represents the atherosclerotic lesion area of an individual mouse and the horizontal line represents the mean for the group. N=23-25/group.

Figure 2.

Increased biglycan and lipid retention in biglycan transgenic mice. Biglycan transgenic (TG) and non-transgenic control (WT) LDL receptor deficient mice were fed an atherogenic Western diet for 4 or 12 weeks. **A.** Aortic roots from mice fed diet for 4 weeks were immunostained for biglycan (red), demonstrating increased medial biglycan in transgenic mice compared to wildtype. Shown are images representative of $n=3-4$ /group, magnified 20X. **B.** Carotid arteries were collected after 12 weeks and immunoblotted for biglycan, showing variable expression. Each lane shows the biglycan content of an individual mouse from the indicated groups, representative of $N=12-18$ /group. Actin is shown as the loading control. TG: transgenic, WT: wildtype. m: male; f: female. **C.** Aortic sinus sections were immunostained for biglycan and biglycan area was quantified using ImageJ software. Each symbol represents the aortic sinus biglycan content of an individual mouse and the horizontal line represents the mean for the group. $N=19-23$ /group. **D.** Carotid biglycan content measured by immunoblot was quantified by densitometry adjusted for actin content (AU: arbitrary units). Atherosclerosis measured in the aortic sinus and carotid biglycan content was analyzed by linear regression; $r=0.539$, $P < 0.0001$. **E.** Atherosclerosis measured in the aortic sinus and aortic sinus biglycan content measured by immunostained area was

analyzed by linear regression; $r=0.330$, $P=0.03$. F. Aortic sinus sections from mice fed diet for 12 weeks were double stained for biglycan (BGN, green) and apoB (red), counterstained with DAPI and visualized by confocal microscopy; in the merge image yellow indicates co-localization. Shown are images representative of $n=5$ /group selected by proximity to the mean biglycan immunostained area in each group, magnified 63X. * indicates the aortic sinus lumen. G. Primary vascular smooth muscle cells from $N=3$ mice/group were incubated with Alexa-fluor labeled LDL for 2h at 4°C and binding quantified by Alexa-fluor intensity normalized to cell count. $P=NS$.